

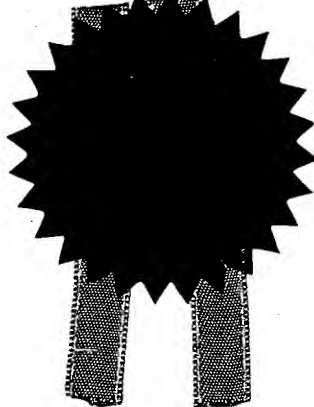
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עם הבקשה לפטנט
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יועצת בכירה לרשם הפטנטים

T/780

לשימוש הלשכה
For Office Use

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בקשה לפטנט

Application for Patent

אני: (שם המבקש, מענו ולגבי גוף מאגד - מקום התאגדותו)
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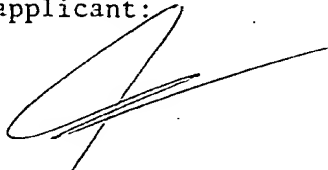
(בעברית)
(Hebrew)

Anti-cytotoxic protein and its Purification

(באנגלית)
(English)

hereby apply for a patent to be granted to me in respect thereof.

מבקש בואת כי ינתן לי עליה פטנט

• בקשה חלוקה - Application of Division		• בקשת פטנט מוסף - Application for Patent Addition		• דרישה דין קדימה Priority Claim	
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90339

Anti-cytotoxic protein and its purification

חלבון אנטיציטוטוקסי וסיהורר

Yeda Research and Development Co. Ltd.

T/780

FIELD OF THE INVENTION

This invention relates to Tumor Necrosis Factor (TNF) Binding Protein (hereinafter TBP-II) and salts, functional derivatives, precursors and active fractions thereof, having the ability to inhibit the cytotoxic effect of TNF and which can be used against the deleterious effects of TNF. The invention also relates to a process for the purification of said TBP-II, to its cloning and its production by recombinant DNA techniques. It further relates to pharmaceutical compositions comprising TBP-II, or salts, functional derivatives, precursors and active fractions thereof, for protecting against the deleterious effects of TNF.

BACKGROUND OF THE INVENTION

Tumor Necrosis Factor (TNF- α) and Lymphotoxin or TNF- β (hereinafter, TNF refers to both TNF- α and TNF- β) are cytokines which have many effects on cells (Wallach, D. (1986) in: Interferon 7 (Ion Gresser, Ed.), pp. 83-122, Academic Press, London, and Beutler, B. and Cerami, A. (1987) New England J. Med. 316: 379-385). Both TNF- α and TNF- β initiate their effects by binding to specific cell surface receptors. Some of the effects are likely to be beneficial to the organism: they may destroy, for example, tumor cells or virus infected cells and augment antibacterial activities of granulocytes. But, quite clearly,

both TNF- α and TNF- β have also effects which can be extensively deleterious. There is evidence that over production of TNF- α can play a major pathogenic role in several diseases. Thus effects of TNF- α , primarily on the vasculature, are now known to be a major cause for symptoms of septic shock (Tracey, K.J. et al. (1986) Science 234: 470-474). In some diseases, TNF may cause excessive loss of weight (cachexia) by suppressing activities of adipocytes and by causing anorexia and TNF- α was thus called cachectin. It was also described as a mediator of the damage to tissues in rheumatic diseases (Beutler, op.cit.) and as a major mediator of the damage observed in graft-versus-host reactions.

There is therefore a necessity in finding out ways to eliminate or antagonize endogenously formed or exogenously administered TNF. Our first attempt in this direction was the development of monoclonal antibodies which neutralize the TNF- α cytotoxic activity and were shown to protect mice against the lethal effect of TNF- α under conditions mimicking elicitation of septic shock (as described in our Israeli Patent Application No. 73883). However, therapy with murine monoclonal antibodies, especially if administered repetitively, may not always be advisable in humans. Therefore the need was felt for development of biological agents which could similarly antagonize the deleterious effects of TNF.

In our Patent Application No. 83878, we disclosed a TNF Inhibitory Protein (hereinafter TBP-I) isolated from human urine and able to inhibit both the binding of TNF to its receptors and the cytotoxic effect of TNF.

As described in said Patent Application No. 83878, TBP-I was purified to homogeneity from human urine by chromatography on CM-Sepharose followed by high performance liquid chromatography (HPLC) on Mono Q and Mono S columns and reversed phase HPLC. The homogeneous TBP-I obtained by said purification had an apparent molecular weight of about 27,000 in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under both reducing and non-reducing conditions, and was shown to protect cells from TNF toxicity at concentrations of a few nanograms per ml and to interfere with the binding of both TNF- α and TNF- β to cells, when applied simultaneously with these cytokines.

Further examination of the mechanism by which TBP-I functions revealed that TBP-I does not interact with the target cell, but rather blocks the function of TNF by binding TNF specifically and thus competes with TNF for the TNF receptor.

Consequently to this finding we attempted an alternative approach for the purification of TBP-I. The urinary proteins or fractions thereof were applied on a column of immobilized TNF and, after removal of unbound proteins, the protein(s) which did bind to TNF were eluted by applying a low pH buffer.

The analysis of the proteins which were eluted from the TNF column after reversed phase HPLC showed the presence of TBP-I, as expected, and, in addition, a second TNF-binding protein was eluted at a different acetonitrile concentration. Although in SDS PAGE analysis the two proteins appeared to have a very similar

molecular size, they could clearly be distinguished from each other: antibodies raised against TBP-I were found not to interact with the second TNF-binding protein (hereinafter TBP-II), and the sequence of amino acids of TBP-II obtained by N-terminal sequence analysis was found to be different from TBP-I. TBP-II shared with TBP-I the ability to protect cells from TNF toxicity.

SUMMARY OF THE INVENTION

The present invention provides TNF Binding Protein, herein designated TBP-II, salts, functional derivatives, precursors and active fractions thereof, which can antagonize the effects of TNF. This antagonism can be determined by measuring reduction of the cytotoxic activity of TNF.

The invention is directed to said TBP-II in substantially purified form, being free of proteinaceous impurities.

The invention also relates to a process for the purification of TBP-II.

The invention further concerns recombinant DNA molecules comprising the nucleotide sequence coding for said protein TBP-II, expression vehicles comprising them and host cells transformed therewith, and to a process for producing TBP-II by culturing said transformant cells in a suitable culture medium.

The TBP-II of the invention and its salts, functional derivatives, precursors and active fractions thereof are for use

as active ingredients of pharmaceutical compositions to protect mammals against the deleterious effects of TNF.

DESCRIPTION OF THE FIGURES

Figure 1 shows the elution pattern of urinary proteins which bind TNF in chromatography on a reversed phase HPLC column. Proteins from crude urine were equilibrated with phosphate-buffered saline (PBS) and applied to a column of immobilized TNF (3.5 mg bound to 0.5 ml of Affigel 10). Bound proteins were eluted at pH 2.5 and then applied to fractionation on an Aquapore RP-300 HPLC column (4.6x30 mm, Brownlee Labs). Elution was performed with a linear gradient of acetonitrile in 0.3% aqueous trifluoroacetic acid (----). Fractions were examined for bioactivity (protection of A9 cells from TNF toxicity) and protein (——) content. Two distinct peaks of protein with bioactivity could be discerned: the first peak eluting at 27% acetonitrile, contains TBP-I, and the second peak, eluting at 31% acetonitrile, contains TBP-II.

Figure 2 demonstrates the lack of immune cross-reactivity between TBP-I and TBP-II. Samples of TBP-I (A), TBP-II (B) and TNF (C), (0.5 µg each) were "dot blotted" on a nitrocellulose paper, which was incubated first for 2h with 10% milk (v/v) in PBS (containing 0.1% sodium azide), then with rabbit antiserum to TBP-I diluted 500 fold in the above solution, and finally for 3h with ¹²⁵I-labelled protein A (6.10⁵ cpm/ml) followed by washing of nonbound radioactivity. Shown is the autoradiography of the blot which demonstrates effective binding of the antibody to TBP-I, but neither to TBP-II nor to TNF.

Figure 3 presents SDS-PAGE analysis of TBP-II, following purification on the Aquapore RP-300 HPLC column, and a demonstration of the ability of this protein to bind TNF. Shown in A is a silver-stained 12% acrylamide gel on which the following samples have been analysed by SDS-PAGE under reducing conditions (in the presence of β -mercaptoethanol). Lanes 1 and 8 (from left to right): molecular weight markers (α Lactalbumin 14.4 kDa; soya bean trypsin inhibitor 20.1 kDa; carbonic anhydrase 30 kDa; ovalbumin 43 kDa; bovine serum albumin 67 kDa and phosphorylase b 94 kDa). Lane 2: 0.5 μ g of TBP-I. Lane 3: sample buffer alone. Lanes 4-7: four consecutive fractions of TBP-II, as eluted from the RP-300 column (0.7-1.0 μ g each).

Shown in B is an autoradiography of a 12% acrylamide gel on which the following samples were analyzed by SDS-PAGE. Lane 1 (from left to right): ^{14}C -labelled molecular weight markers (Amersham Catalogue No. CFA.626). Lane 2: ^{125}I -labelled TBP-II (10^4 CPM). Lane 3: ^{125}I -labelled TBP-II which was examined for ability to rebind TNF. It was applied to TNF-coated PVC plates and then, after washing out non-bound proteins, it was eluted with SDS (10^4 CPM). Lane 4: control for the experiment of lane 3: ^{125}I -labelled TBP-II applied in presence of excess TNF (1 μ g), which largely blocked the binding of the labelled protein to the plate). Lane 5: ^{125}I -labelled TBP-I (10^4 CPM).

Figure 4 shows that both TBP-I and TBP-II are capable of binding TNF even after exposure to the denaturing effect of SDS. The following samples were analyzed by SDS-PAGE on 12% acrylamide

gel. Lane 1: molecular weight markers as in Fig.3 (run in the presence of β -mercaptoethanol). Lane 2: sample buffer alone. Lanes 3 and 4: samples of 0.5 μ g of TBP-I and TBP-II, respectively. Lane 5: 0.5 μ g of a contaminant eluted from the TNF affinity column as control (Fraction 13 eluted from the RP300 column in the experiment presented in Fig. 1). Lanes 3 to 5 were all analyzed in the absence of β -mercaptoethanol. The proteins were then blotted into nitrocellulose paper, with the use of the Biorad Western blotting device. After blocking the paper with milk (as in Fig. 2), it was incubated for 3h at room temperature with 125 I-labelled TNF ($6.5 \cdot 10^5$ CPM/ml in 10% milk in PBS). It was then washed 5 times with PBS containing 0.05% Tween 20 and 0.02% azide and exposed to autoradiography.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides TNF Binding Protein TBP-II and salts, functional derivatives, precursors and active fractions thereof, having the ability to inhibit the cytotoxic effect of TNF.

It was found according to the present invention that TBP-II is able to inhibit the biological activity of TNF and thus the inhibition of this cytokine, by the TBP-II, is encompassed by the present invention.

The TBP-II of the invention may be found in human urine. The substantially purified protein, which is substantially free of proteinaceous impurities, has a molecular weight of about 30 kDa when analyzed by SDS PAGE under reducing conditions and it moves

as a single peak on reversed-phase HPLC. Its activity is determined by its ability to inhibit the cytotoxic effect of TNF- α on murine A9 cells.

It is further characterized by the following sequence obtained by N-terminal sequence analysis of the protein:

Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr

or by the sequence:

Phe-Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr

or by the sequence:

Val-Ala-Phe-Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr

These sequences were determined in three consecutive fractions in the elution of TBP-II from the RP-300 HPLC column as described in Fig. 1 (fractions 26, 27 and 28).

The polypeptides comprising all the above sequences are included in the invention and referred to as TBP-II. Any other polypeptide in which one or more amino acids in the structure of natural TBP-II are deleted or replaced with other amino acids is also encompassed by the present invention as long as they have human TBP-II activity.

As used herein the term 'salts' refers to both salts of carboxyl groups and to acid addition salts of amino groups of the protein molecule. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine

and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid.

"Functional derivatives" as used herein covers derivatives which may be prepared from the functional groups which occur as side chains on the residues or the N- or C- terminal groups, by means known in the art, and are included in the invention as long as they remain pharmaceutically acceptable, i.e. they do not destroy the activity of the protein and do not confer toxic properties on compositions containing it.

These derivatives (may, for example) include aliphatic esters of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives of free amino groups of the amino acid residues formed with acyl moieties (e.g. alkanoyl or carbocyclic aroyl groups) or O-acyl derivatives of free hydroxyl group (for example that of seryl or threonyl residues) formed with acyl moieties.

"Precursors" are compounds formed prior to, and converted into, TBP-II in the animal or human body.

As "active fractions" of the substantially purified protein, the present invention covers any fragment or precursors of the polypeptide chain of the protein molecule alone or together with

associated molecules or residues linked thereto, e.g. sugar or phosphate residues, or aggregates of the protein molecule or the sugar residues by themselves, provided said fraction has the ability to inhibit the cytotoxic effect of TNF on cells. As with TBP-I, it was observed that TBP-II decreases the ^{125}I -TNF- α binding to the cells only when ^{125}I -TNF- α and TBP-II are applied together on cells and not when TBP-II is first applied on cells and then removed prior to the application of TNF- α . This indicates that the interference with TNF- α binding to cells is not due to an effect of the TNF Binding Protein TBP-II on the cells, but it rather reflects some kind of interaction between the protein of the invention and TNF- α .

1. BIOASSAY FOR TBP-II

The same bioassay developed for TBP-I and based on the cytotoxic effect of TNF on cycloheximide (CHI)-sensitized cells and its quantitation by the neutral-red uptake method, as described in Wallach, D. (1984) J. Immunol. 132:2464-2469, is used also in the present application to measure TBP-II bioactivity.

- Samples to be tested for the presence of the protein were diluted two-fold serially, at 4°C, in Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM), and an equal volume of the same medium containing 40 Ug/ml TNF- α and 400 $\mu\text{g}/\text{ml}$ cycloheximide (CHI) is added thereto.
- Murine A9 cells were seeded in 96-well flat-bottom microtiter plates ($1,5 \times 10^4$ cells/ well) with 100 μl DMEM-CS (DMEM

containing 5% fetal calf serum and 5% calf serum).

- 100 μ l aliquots of the serially diluted protein TNF- α -CHI mixtures were applied to each well and the cells were further incubated for 14 hours.
- Viability of the cells was determined by incubation with neutral red for 2 hours, washing away excess dye, extracting the neutral red that was taken up by the cells with Sorenson's citrate buffer-ethanol mixture, and quantitating it colorimetrically at 570 nm with a Microelisa Auto-reader.
- 1 U/ml of TNF inhibitor activity was defined as the dilution factor giving a statistically significant protection from TNF killing ($p < 0.05$).

2. PURIFICATION OF TBP-II

In the preferred embodiment of the invention, the substantially purified protein of the invention is produced by a process which comprises:

- X a) recovering the crude protein fraction from a dialyzed concentrate of human urine;
- b) subjecting said crude protein fraction of step (a) to affinity purification on a column of immobilized TNF;
- c) applying said affinity purified active TNF Binding Proteins from step (b) to reversed phase HPLC to obtain substantially purified active fractions of the TNF Binding Proteins defined by their ability to inhibit the cytotoxic effect of TNF;

- d) separating the substantially purified proteins of step (c) having a molecular weight of about 30 kDa on SDS PAGE under reducing conditions, moving as a single peak on reversed phase HPLC and having the ability to inhibit the cytotoxic effect of TNF; and
- e) recovering the fractions eluting at 31% acetonitrile and containing substantially purified TBP-II.

2.1 Preparation of the urine concentrate

A pool of 200 l male urine from healthy donors was subjected to micro-filtration on a Pellicon membrane with a pore size of 0.45 μ m. The filtrate was concentrated by ultrafiltration using a Pellicon membrane with a molecular weight cut off of 10 kDa to a final volume of 500 ml. The concentrate was dialyzed against phosphate buffered saline containing 1 mM benzamidine and 0.1% sodium azide.

2.2 Affinity purification of TNF binding proteins on column of immobilized TNF

Recombinant TNF- α was brought to a concentration of 7.2 mg/ml, then equilibrated with PBS containing 0.02% sodium azide and coupled to Affigel 10 (3.6 mg to 0.5 ml beads). The concentrate of urinary proteins of step 2.1 was applied to a column constructed from the beads of the immobilized TNF at a flow rate of 0.2 ml/min. at 4°C. Unbound proteins were washed with PBS and the bound proteins were then eluted by applying a solution of 20 mM citric acid, 100 mM NaCl. The eluted protein was monitored for protein and for bioactivity (inhibition of TNF toxicity).

2.3 Reversed-phase high pressure liquid chromatography (HPLC)

The reversed-phase HPLC column Aquapore RP 300 4.6 x 30 mm (Brownlee Labs) was prewashed with 0.3% aqueous trifluoroacetic acid (TFA) (Buffer F) until a stable baseline was obtained by the fluorescamine detection system. The active fractions which were eluted from the affinity TNF columns of step 2.2 were pooled and injected in one 1.6 ml portion onto the column. The column was run with Buffer F at a flow rate of 0.5 ml/minute until the fluorometer did not detect any protein. The column was then eluted at a flow rate of 0.5 ml/minute, with a 0-20% linear gradient of acetonitrile in Buffer F for 5 minutes, followed by a 20-50% linear gradient of acetonitrile for 60 minutes and finally a 50% to 80% linear acetonitrile gradient for 5 minutes. The column was then washed for 15 minutes with 80% acetonitrile. Fractions of 0.5 ml were collected and tested for protein content and for bioactivity. As shown in figure 1, the active proteins were found to elute as two distinct protein peaks, in fractions corresponding to about 27% acetonitrile (TBP-I) and about 31% acetonitrile (TBP-II).

2.4 SDS-PAGE

In order to monitor the result of the purification, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed (Figure 3) according to the method of Laemmli U.K. et al. (1970) Nature 227:680. Samples of the active fractions eluting from the reversed phase HPLC, were mixed with 3 x concentrated sample buffer containing 6% SDS (w/v) and 15% v/v

β -mercaptoethanol and loaded on a 15% acrylamide gel. As a reference for molecular weight, a mixture of molecular weight markers (α lactalbumin 14.4 kDa, soya bean trypsin inhibitor 20.1 kDa, carbonic anhydrase 30 kDa, ovalbumin 43 kDa, bovine serum albumin 67 kDa, and phosphorylase b. 94 kDa) was treated as above and loaded on lanes 1 and 8. A blank with sample buffer was run on lane 3. The gel was run at 160 volt and the protein bands were visualized by silver staining (Oakley, B.R. et al. Anal. Biochem. 105:361) As shown in figure 3, each of the two purified TNF Binding Proteins moved as a single band, both with an apparent molecular weight of 30 kDa (Lane 2 - TBP-I; Lanes 4-7 - TBP-II).

2.5 N-Terminal Sequence Analysis

Samples of the substantially purified TBP-II of the invention (1-5 μ g, 50-200 pmol each) were applied to pretreated, biobrene-coated glass-fiber discs. The dried discs were subjected to repetitive cycles of Edman degradation in an automated pulsed liquid gas phase protein microsequencer (Model 475) with an on-line HPLC PTH-amino acid analyzer (Model 120) and a data acquisition and processing unit Model 900, (all from Applied Biosystems Inc. Foster City, CA, U.S.A.). The computer-derived sequence was compared with the raw data and was corrected when necessary. Altogether three separate analyses were performed in order to confirm the sequence data. The initial yield was over 40%, indicating that the major protein in the preparation (the 30 kDa band) is related to the resulting sequence.

The N-terminal sequencing of the TBP-II gave the following amino acid sequences:

Val-Ala-Phe-Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr

and:

Phe-Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr

and:

Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr

A computerized search in the National Biomedical Research Foundation protein library (update No. 16) by the FASTP method did not reveal a significant homology to any known protein.

3. GENETIC ENGINEERING OF THE TBP-II

This invention further concerns DNA molecules comprising the nucleotide sequence coding for the TBP-II of the invention, replicable expression vehicles containing said DNA molecules, hosts transformed therewith and the TBP-II produced by expression of such transformed hosts. The term "DNA molecules" includes genomic DNA, cDNA, synthetic DNA and combinations thereof.

The cloning of TBP-II may be carried out by different techniques. According to one approach, specific antibodies (polyclonal or monoclonal) to the TBP-II are produced and used to clone the TBP-II cDNA. This approach is comprised of the following three steps:

- a) Preparation of antibodies: The antibodies to TBP-II can be produced either by using the substantially purified TBP-II of the present invention or by using one or more synthetic peptides identical to the known sequence of the protein, e.g.

the N-terminal protein sequence, or by fusing one of the possible nucleotide sequences deduced from the amino acid sequence of TBP-II to the gene coding for Protein A and expressing the fused Protein A - TBP-II in E. coli.

For obtaining polyclonal antibodies, the substantially purified TBP-II or the synthetic peptides linked to a carrier protein are injected into rabbits. For the production of monoclonal antibodies, the fused Protein A - TBP-II synthetic gene is expressed in E. coli, the fused protein obtained is purified by affinity chromatography on IgG Sepharose column and injected into mice. Alternatively, the substantially purified TBP-II of the present invention is injected into mice.

b) Screening of TBP-II producing cells

Anti-TBP-II antibodies are used to search for cells producing TBP-II by immunofluorescence or by Western blot.

c) Preparation of cDNA from producing cells

mRNA is extracted from TBP-II producing cells and cDNA is prepared by the use of reverse transcriptase. The cDNA is cloned in an expression vector such as λ gt 11 and screened by the use of the antibodies. The λ gt 11 expression vector can be used for insertion of DNA up to 7 kb in length at a unique EcoRI site 53 bases upstream from the β -galactosidase termination codon. Therefore, foreign sequences DNA may be inserted into this site and expressed under appropriate

conditions as fusion proteins. The λ gt 11 expression vector is particularly useful for the construction of cDNA libraries to be screened with antibody probes (Huynh, T.V. et al. in: David Glover (ed.), DNA Cloning Techniques: A Practical Approach, IRL Press, Oxford (1984) pp. 49-78), as outlined here.

Following another approach, a synthetic oligonucleotide or a mixture of synthetic oligonucleotides, whose sequence is derived from the sequence of a fragment of the protein, e.g., the N-terminal amino acid sequence of TBP-II are produced and this oligonucleotide or the mixture of oligonucleotides are used as a probe for cloning the cDNA or the genomic DNA coding for TBP-II.

The genomic DNA may or may not include naturally occurring introns. It may be obtained, for example, by extraction from suitable cells and purification by means well known in the art. Suitable DNA preparations, such as human genomic DNA, are enzymatically cleaved by restriction enzymes, or randomly sheared, and the fragments inserted into appropriate recombinant vectors to form a gene library. Such vectors can then be screened with synthetic oligonucleotide probes in order to identify a sequence coding for TBP-II.

Alternatively, mRNA is isolated from a cell which expresses the protein of the invention and used to produce cDNA by means well known in the art. This cDNA, after conversion to the double-stranded form, may be cloned and the resulting clone screened with an appropriate probe for cDNA coding for the desired

sequences. Once the desired clone has been isolated, the cDNA may be manipulated in substantially the same manner as the genomic DNA. However, with cDNA there will be no introns or intervening sequences.

In order to synthesize the oligonucleotides to be used as probes, it is possible either to perform sequence analysis of the intact TBP-II or to obtain peptide fragments thereof and to characterize their amino acid sequence. In order to obtain peptide fragments, purified protein preparations are subjected to fragmentation, e.g. by digestion with proteases such as trypsin, chymotrypsin or papain by methods well known in the art (Oike, Y. et al. (1982) J. Biol. Chem. 257:9751-9758). The peptide fragments produced by digestion are separated by reverse phase HPLC and sequenced by automatic amino acid sequencing techniques.

As already described, the following sequences of amino acids were obtained by N-terminal sequence analysis of the protein:

Val-Ala-Phe-Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr

and:

Phe-Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr

and:

Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr

Once one or more suitable peptide fragments have been sequenced or a partial sequence of the protein is determined, the DNA sequences capable of encoding them are examined. Due to the degeneration of the genetic code, more than one codon may be used to encode a particular amino acid and one or more different

oligonucleotides can be produced, each of which would be capable of encoding the TBP-II peptide fragments (Watson, J.D., in: Molecular Biology of the Gene, 3rd ed., W.A. Benjamin, Inc. Menlo Park, CA (1977), pp. 356-357). However, only one member of the set contains the nucleotide sequence that is identical to the nucleotide sequence of the gene. Its presence within the set and its capability to hybridize to DNA even in the presence of the other members of the set, makes it possible to employ the unfractionated set of oligonucleotides in the same manner in which one would employ a single oligonucleotide to clone the gene that encodes the peptide. The use of such oligonucleotide or set of oligonucleotides containing the theoretical "most probable" sequence capable of encoding the TBP-II gene fragments (following the "codon usage rules" disclosed by Lathe, R., et al. (1985) J. Molec. Biol. 183:1-12) permits to identify the sequence of a complementary oligonucleotide or set of oligonucleotides which is capable of hybridizing to the "most probable" sequence encoding the TBP-II or at least a portion thereof, or a set of such sequences. This oligonucleotide containing such a complementary sequence may then be synthesized and employed as a probe to identify and isolate the gene of the TBP-II of the invention (Maniatis, T. et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1982)).

Once a suitable oligonucleotide, or set of oligonucleotides, which is capable of encoding a fragment of the TBP-II gene (or which is complementary to such an oligonucleotide, or set of oligonucleotides) is identified using the above-described

procedure, it is synthesized and hybridized to a DNA or, preferably, to a cDNA preparation derived from cells which are capable of expressing the desired gene, preferably after the source of cDNA has been enriched for the desired sequences, e.g. by extracting RNA from cells which produce high levels of the desired gene and then converting it to the corresponding cDNA by employing the enzyme reverse transcriptase.

Procedures for hybridization of nucleic acids are common knowledge and are disclosed, for example, in Maniatis, T., Molecular Cloning: A Laboratory Manual, op. cit. and in Haymes, B.T., et al., Nucleic Acid Hybridization: A Practical Approach, IRL Press, Oxford, England (1985). By hybridization with the above nucleotide or set of oligonucleotides probes, it is possible to identify in a cDNA or genomic library, the DNA sequences capable of such hybridization and they are then analyzed to determine to what extent they contain encoding sequences for the TBP-II of the invention.

By the same or similar techniques it has been possible to successfully clone the genes for several human proteins, such as the tissue-type plasminogen activator (Pennica, D. et al. (1983) Nature 301:214-221).

The DNA molecules coding for the TBP-II of the invention, obtained by the above described methods, are then inserted into appropriately constructed expression vectors by techniques well known in the art (see Maniatis et al., op cit.). Double-stranded

cDNA is linked to plasmid vectors by homopolymeric tailing or by restriction linking involving the use of synthetic DNA linkers or blunt-ended ligation techniques. DNA ligases are used to ligate the DNA molecules and undesirable joining is avoided by treatment with alkaline phosphatase.

In order to be capable of expressing a desired protein, an expression vector should comprise also specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding for the desired protein in such a way as to permit gene expression and production of the protein. First, in order for the gene to be transcribed, it must be preceded by a promoter recognizable by RNA polymerase, to which the polymerase binds and thus initiates the transcription process. There are a variety of such promoters in use, which work with different efficiencies (strong and weak promoters). They are different for prokaryotic and eukaryotic cells.

The promoters that can be used in the present invention may be either constitutive, for example, the int promoter of bacteriophage, the bla promoter of the β -lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene of pPR325, etc., or inducible, such as the prokaryotic promoters including the major right and left promoters of bacteriophage (P_L and P_R), the trp, recA, lacZ, lacI, ompF and gal promoters of E. coli, or the trp-lac hybrid promoter, etc. (Glick, B.R. (1987) J. Ind. Microbiol. 1:277-282).

Besides the use of strong promoters to generate large quantities of mRNA, in order to achieve high levels of gene expression in prokaryotic cells, it is necessary to use also ribosome-binding sites to ensure that the mRNA is efficiently translated. One example is the Shine-Dalgarno sequence (SD sequence) appropriately positioned from the initiation codon and complementary to the 3'-terminal sequence of 16S RNA.

For eukaryotic hosts, different transcriptional and translational regulatory sequences may be employed, depending on the nature of the host. They may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus, or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Examples are the TK promoter of Herpes virus, the SV40 early promoter, the yeast gal4 gene promoter, etc. Transcriptional initiation regulatory signals may be selected which allow for repression and activation, so that expression of the genes can be modulated.

The DNA molecule comprising the nucleotide sequence coding for the TBP-II of the invention and the operably linked transcriptional and translational regulatory signals is inserted into a vector which is capable of integrating the desired gene sequences into the host cell chromosome. The cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for prototrophy to an auxotrophic host, biocide

resistance, e.g., antibiotics, or heavy metals, such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of single chain binding protein mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama, H., (1983) Mol. Cel. Biol. 3:280.

In a preferred embodiment, the introduced DNA molecule will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Preferred prokaryotic vectors include plasmids such as those capable of replication in E. coli, for example, pBR322, ColE1, pSC101, pACYC 184, etc. (see Maniatis et al., Molecular Cloning: A Laboratory Manual, op.cit.); Bacillus plasmids such as pC194, pC221, pT127, etc. (Gryczan, T., The Molecular Biology of the Bacilli, Academic Press, NY (1982), pp. 307-329); Streptomyces plasmids including pIJ101 (Kendall, K.J. et al., (1987) J.

Bacteriol. 169:4177-4183); Streptomyces bacteriophages such as ØC31 (Chater, K.F. et al., in: Sixth International Symposium on Actinomycetales Biology, Akademiai Kiado, Budapest, Hungary (1986), pp. 45-54), and Pseudomonas plasmids (John, J.F., et al. (1986) Rev. Infect. Dis. 8:693-704), and Izaki, K. (1978) Jpn. J. Bacteriol. 33:729-742).

Preferred eukaryotic plasmids include BPV, vaccinia, SV40, 2-micron circle, etc., or their derivatives. Such plasmids are well known in the art (Botstein, D., et al. (1982) Miami Wint. Symp. 19:265-274; Broach, J.R., in: The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 445-470 (1981); Broach, J.R., (1982) Cell 28:203-204; Bollon, D.P., et al. (1980) J. Clin. Hematol. Oncol. 10:39-48; Maniatis, T., in: Cell Biology: A Comprehensive Treatise, Vol. 3: Gene Expression, Academic Press, NY, pp. 563-608 (1980)).

Once the vector or DNA sequence containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, etc.

Host cells to be used in this invention may be either prokaryotic or eukaryotic. Preferred prokaryotic hosts include bacteria such as E. coli, Bacillus, Streptomyces, Pseudomonas, Salmonella,

Serratia, etc. The most preferred prokaryotic host is E. coli. Bacterial hosts of particular interest include E. coli K12 strain 294 (ATCC 31446), E. coli X1776 (ATCC 31537), E. coli W3110 (F⁻, lambda⁻, prototropic (ATCC 27325)), and other enterobacterium such as Salmonella typhimurium or Serratia marcescens and various Pseudomonas species. Under such conditions, the protein will not be glycosylated. The prokaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

Preferred eukaryotic hosts are mammalian cells, e.g., human, monkey, mouse and chinese hamster ovary (CHO) cells, because they provide post-translational modifications to protein molecules including correct folding or glycosylation at correct sites. Also yeast cells can carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e. pre-peptides).

After the introduction of the vector, the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the desired TBP-II or a fragment thereof. The expressed protein is then isolated and purified in accordance with the purification method described in the present application (section 2 supra) or by any other conventional

procedure involving extraction, precipitation, chromatography, electrophoresis, or the like.

A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using anti-TBP-II monoclonal antibodies, which are produced and immobilized on a gel matrix contained within a column. Impure preparations containing the recombinant protein are passed through the column. The protein will be bound to the column by the specific antibody while the impurities will pass through. After washing, the protein is eluted from the gel by a change in pH or ionic strength.

The monoclonal antibodies used in the present invention can be prepared using conventional hybridoma technique (Kohler et al. (1975) *Nature* 256:495; Kohler et al. (1976) *Eur. J. Immunol.* 6:511). In general, such procedures involve immunizing an animal with the desired purified protein antigen or with a synthetic peptide having the N-terminal sequence of the desired protein conjugated to a suitable carrier, such as bovine serum albumin. Spleen cells of such animals are isolated and fused with a suitable myeloma cell line. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium and then cloned. The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding TBP-II. After identification, the desired clone can be grown in bulk, either in suspension culture or in ascitic fluid by injecting the cells into the peritoneum of suitable host mice.

The monoclonal antibodies produced by said hybridomas, after purification and immobilization, are very efficient for the purification of the TBP-II in affinity purification procedure using an immuno-adsorbent column.

5. UTILITY AND COMPOSITIONS

The TBP-II and salts, functional derivatives, precursors and active fractions thereof are indicated for antagonizing the deleterious effects of TNF in mammals, i.e. for treating conditions wherein excess of TNF is formed endogenously or is exogenously administered.

The present invention further relates to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and the TBP-II of the invention or its salts, functional derivatives, precursors or active fractions thereof, as active ingredient. These compositions may be used in any condition where there is an over production of endogenous TNF, such as in cases of septic shock, cachexia, graft-versus host reactions, autoimmune diseases like rheumatoid arthritis, etc. The way of administration can be via any of the accepted modes of administration for similar agents and will depend on the condition to be treated, e.g., intravenously in case of septic shock or local injection in case of rheumatoid arthritis (for example, into the knee), or continuously by infusion, etc. The compositions may also be used in cases of TNF intoxication caused by exogenous administration of excessive amounts (overdoses) of TNF.

The pharmaceutical compositions of the invention are prepared for administration by mixing the protein or its derivatives with physiologically acceptable carriers, stabilizers and excipients, and prepared in dosage form, e.g. by lyophilization in dosage vials. The amount of active compound to be administered will depend on the route of administration, the disease to be treated and the condition of the patient. Local injection in case of inflammatory conditions of rheumatoid arthritis will require less TBP-II on a body weight basis than will intravenous infusion in case of septic shock.

Claims

1. Tumor Necrosis Factor (TNF) Binding Protein, herein designated TBP-II, salts, functional derivatives, precursors and active fractions thereof, having the ability to inhibit the cytotoxic effect of TNF.
2. The TNF Binding Protein TBP-II of claim 1 in substantially purified form.
3. The TNF Binding Protein TBP-II of claim 1 or 2 having a molecular weight of about 30 kDa when the substantially purified protein is analyzed by SDS-PAGE under reducing conditions.
4. The TNF Binding Protein TBP-II of claim 1 or 2 moving as a single peak on reversed phase high performance liquid chromatography (HPLC).
5. The TNF Binding Protein TBP-II according to any of the preceding claims having the ability to inhibit the cytotoxic effect of TNF- α on murine A9 cells.
6. The TNF Binding Protein TBP-II according to any of the preceding claims which contains the following amino acid sequence obtained by N-terminal analysis:

Val-Ala-Phe-Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr

7. The TNF Binding Protein TBP-II according to any of the preceding claims which contains the following amino acid sequence obtained by N-terminal analysis:

Phe-Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr

8. The TNF Binding Protein TBP-II according to any of the preceding claims which contains the following amino acid sequence obtained by N-terminal analysis:

Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr

9. A process for the production of substantially purified TBP-II Protein which comprises:

- (a) recovering the crude protein fraction from a dialyzed concentrate of human urine;
- (b) subjecting said crude protein fraction of step (a) to affinity chromatography on a column of immobilized TNF to obtain purified active fractions of TNF Binding Proteins defined by their ability to inhibit the cytotoxic effect of TNF;
- (c) applying said purified active fractions of the TNF Binding Proteins from step (b) to reversed phase high pressure liquid chromatography (HPLC) to obtain substantially purified active fractions of TNF Binding Proteins defined by their ability to inhibit the cytotoxic effect of TNF; and

(d) recovering the substantially purified TBP-II protein of step (c), said protein having a molecular weight of about 30 on SDS PAGE under reducing conditions, moving as a single peak in the fraction corresponding to about 31% acetonitrile on reversed phase HPLC and having the ability to inhibit the cytotoxic effect of TNF.

10. A process according to claim 9 wherein the activity of the fractions in steps (b), (c) and (d) is defined by the ability of the TNF Binding Proteins to inhibit the cytotoxic effect of TNF- α on murine A9 cells.

11. The TNF Binding Protein TBP-II according to claim 1 or 2 produced by the process of claim 9 or 10.

12. The human TNF Binding Protein TBP-II of claim 2.

13. The human TNF Binding Protein TBP-II of claim 6.

14. The human TNF Binding Protein TBP-II of claim 7.

15. The human TNF Binding Protein TBP-II of claim 8.

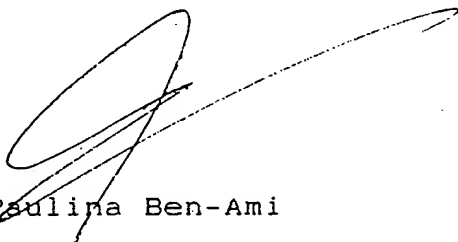
16. The TNF Binding Protein TBP-II of claim 1 which is a recombinant protein.

17. A DNA molecule comprising the nucleotide sequence coding for the TNF Binding Protein TBP-II of claim 1.

18. The DNA molecule of claim 17 wherein the nucleotide sequence is selected from the group consisting of genomic DNA and cDNA.
19. A replicable expression vehicle comprising the DNA molecule of claim 17 and capable, in a transformant host cell, of expressing the TNF Binding Protein TBP-II defined in any one of claims 1 to 8 or 16.
20. A host cell transformed with the replicable expression vehicle of claim 19.
21. A prokaryotic host cell according to claim 20.
22. A eukaryotic host cell according to claim 20.
23. A process for producing TNF Binding Protein TBP-II comprising the steps of: (a) culturing a transformant host cell according to claim 20 in a suitable culture medium, and (b) isolating said TNF Binding Protein TBP-II.
24. TNF Binding Protein TBP-II produced by the process of claim 23.
25. A pharmaceutical composition comprising TNF Binding Protein TBP-II and/or salts, functional derivatives, precursors or active fractions thereof as active ingredient together with a pharmaceutically acceptable carrier.

26. TNF Binding Protein TBP-II and salts, functional derivatives, precursors or active fractions thereof, for use in antagonizing the deleterious effect of TNF in mammals.
27. TNF Binding Protein TBP-II and salts, functional derivatives, precursors or active fractions thereof, for use in the treatment of conditions wherein excess of TNF is formed endogenously or is exogenously administered.

For the Applicants:



Paulina Ben-Ami
Patent Attorney

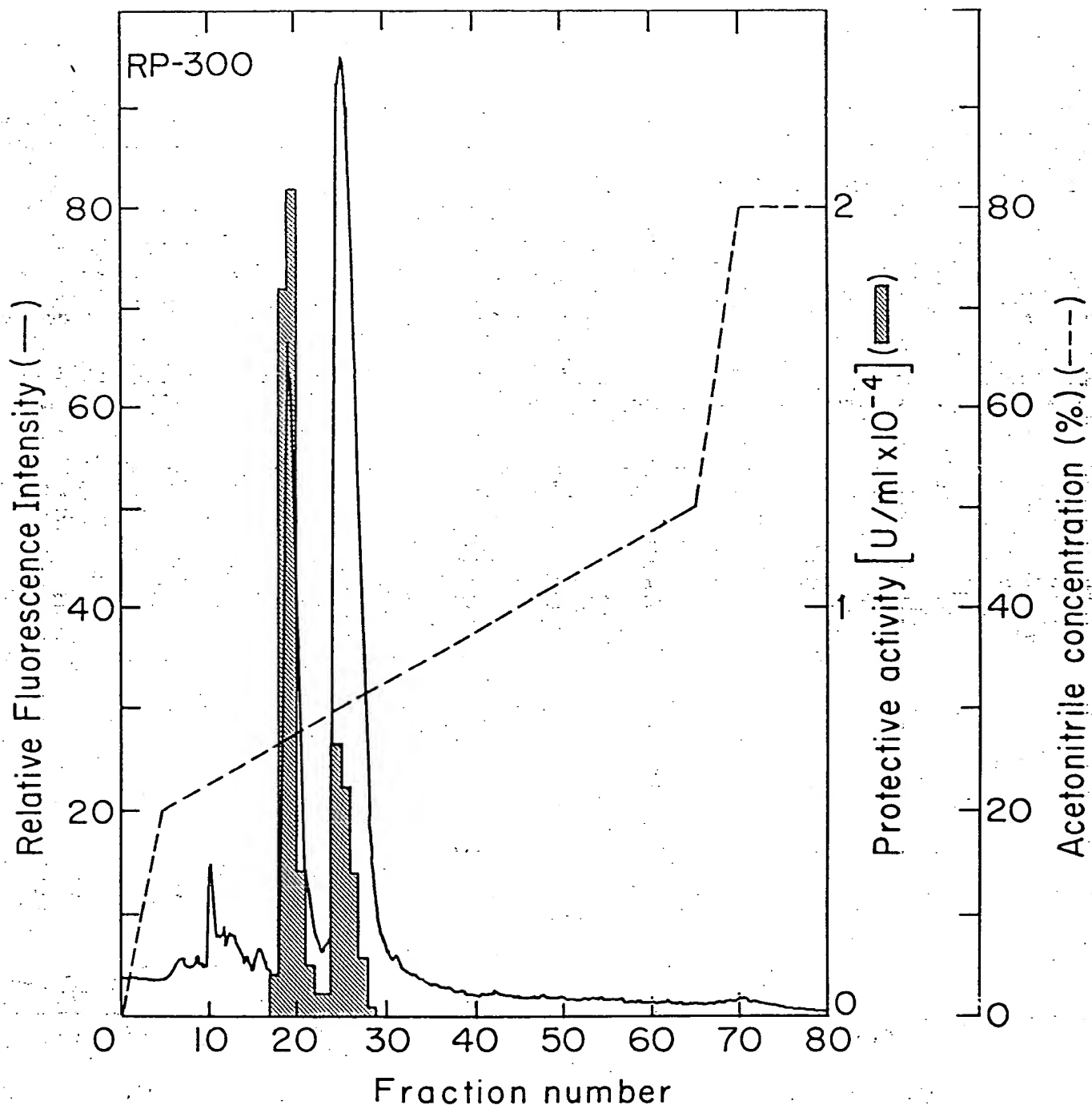


FIG. 1

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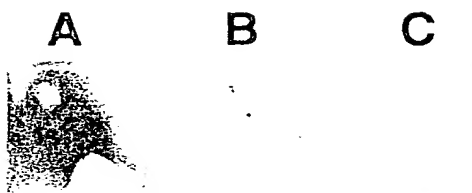


FIG. 2

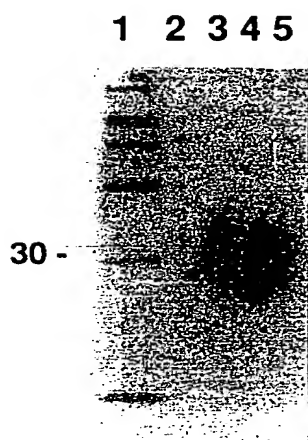


FIG. 4

A

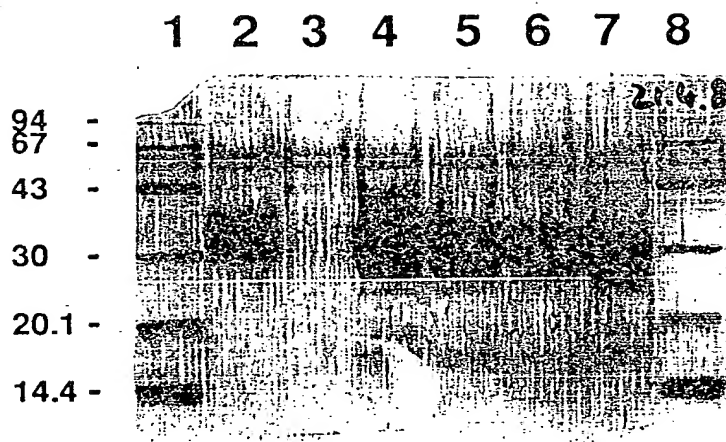


FIG. 3A

B

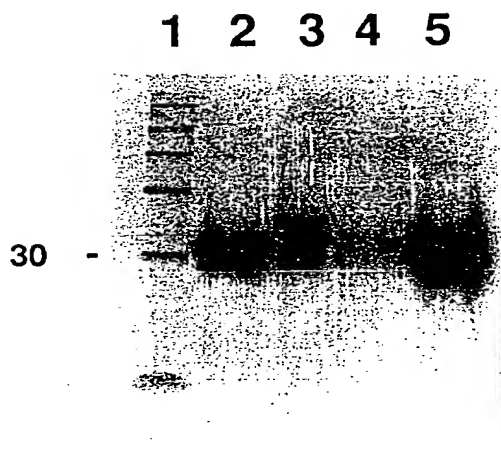


FIG. 3B

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